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Maillard crosslinking of food proteins II: the reactions of glutaraldehyde, formaldehyde and glyceraldehyde with wheat proteins in vitro and in situ

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Abstract

As part of a study of the effects of the Maillard reaction on food texture, we have established, in the preceding paper, the capacities of formaldehyde, glyceraldehyde and glutaraldehyde to crosslink a model protein. In this paper, we validate our model study using wheat proteins, both in vitro and in situ. All three molecules were found to crosslink all fractions of wheat proteins (albumins and globulins, gliadins, SDS-soluble glutenins and SDS-insoluble glutenins) in vitro, with glutaraldehyde being the most reactive. Of the four fractions of wheat proteins, gliadins proved the least susceptible to Maillard crosslinking. Crosslinking was accompanied by a loss of lysine residues in all cases. In situ, only glutaraldehyde underwent protein crosslinking, and the reaction was specific to the albumin and globulin fraction. In the following paper, we explore the effects that this specific crosslinking of albumins and globulins in dough has on the texture of bread and croissants.

Keywords: Protein crosslinking; Wheat proteins; Maillard; Glycation; Cereal foods

1. Introduction

Wheat is unique amongst the cereals in that its flour, when mixed with water, forms a viscoelastic dough capable of producing bread upon baking (Bushuk, 2000). The functional properties of dough are largely attributed to the characteristics of the endosperm storage proteins in the flour, often referred to as gluten proteins (Wrigley, Andrews, Bekes, Gras, Gupta, MacRitchie et al., 2000). It is perhaps not surprising, therefore, that crosslinking of these glutens has an influence on the properties of the dough and the subsequent baked product.

Traditionally, the only type of protein crosslinking that was considered in the context of wheat dough was disulfide bonding (Kaufman, Hoseney, & Fennema, 1986). However, previous work in this laboratory has established that non-disulfide crosslinks, specifically those introduced by the enzyme transglutaminase, can also influence dough properties (Gerrard, Fayle, Wilson, Newberry, Ross, & Kavale, 1998; Gerrard, Newberry, Ross, Wilson, Fayle, & Kavale, 2000; Gerrard, Fayle, Brown, Sutton, Simmons, & Rasiah, 2001). Tilley has also espoused the importance of dityrosine crosslinks in dough (Tilley, Benjamin, Bagorogoza, Okot-Kotber, Prakash, & Kwen, 2001). Thus it seems that the chemical nature of the protein crosslink itself is not important, and novel methods to introduce crosslinks have the potential to improve cereals processing and the quality of cereal food products.

The Maillard reaction is known to result in protein crosslinking, and yet its effects on wheat proteins, and whether these have a functional influence on dough, have received little attention. In this paper, we explore the potential of glutaraldehyde, formaldehyde and glyceraldehyde, shown to crosslink a model protein at varying rates (see preceding paper), to crosslink wheat proteins. The crosslinking experiments were performed both in vitro, on purified wheat proteins, and in situ in the dough itself, in order to assess the differential reactivity of the compounds in the test tube and within an actual foodstuff.

Wheat proteins are generally classified on the basis of their solubility. The sequential extraction of wheat proteins, in different solutions, was pioneered over 90 years ago (Osborne, 1907), and still forms the basis of wheat

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Nomenclature

RNAse A	ribonuclease A
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
OPA	*o*-phthaldialdehyde

protein classification and many other modern fractionation procedures. The Osborne solubility fractions consist of four main classes of wheat protein: the albumins (water-soluble), globulins (soluble in dilute salt solution), gliadins (soluble in 70% ethanol), and the glutenins (partially soluble in dilute acids or alkali). Many modifications of the fractionation procedure have been developed; in this study, protein was extracted from wheat dough using a variation of the methods of Hay and Sutton (Hay, 1993) and of Batey, Gupta, and MacRitchie (1991).

2. Materials and methods

2.1. General

Unless otherwise stated, all materials were obtained from Sigma Chemical Company (St Louis, MO, USA). Bradford reagent was obtained from BioRad (NSW, Australia). Commercial bread baking flour was purchased from Champion Flourmill and stored at −18 °C.

2.2. Dough preparation and protein extraction

Doughs were made according to the following recipe: flour (125 g) and salt (2.5 g) were weighed into a metal tin. Water (80 mL), with a temperature of approximately 10 °C, was poured into a measuring cylinder. Where required, glutaraldehyde, glyceraldehyde and formaldehyde were added to the water and thoroughly mixed, immediately prior to addition. The flour and salt were added to the mixing bowl of a variable speed mixer, followed by the water. The mixing curve of each dough was monitored and the mixer turned off when the optimum degree of mixing had been reached. The dough was then removed from the mixer, frozen in liquid nitrogen, and stored at −18 °C. Doughs were freeze-dried and ground into a flour-like powder. Samples (100 mg) were weighed into 1.5 ml Eppendorf tubes prior to extraction. Wheat proteins were fractionated as previously described (Gerrard et al., 2001).

2.3. In vitro incubations

Wheat protein extracts were thawed overnight to allow the protein, especially the SDS-insoluble glutenin, to solubilise fully. Samples of each wheat protein fraction were pipetted into labelled Eppendorf tubes. Carbonyl compound solutions were made to the appropriate concentration. Glutaraldehyde and formaldehyde solutions were diluted using distilled water. The pH was then adjusted to 7 with HCl, and the final volume made up with distilled water. 10 μl (glutaraldehyde, formaldehyde) or 20 μl (glyceraldehyde) aliquots were added to each Eppendorf and the mixture vortexed and incubated at 37 °C. The final volume in each Eppendorf tube was 500 μl. Each sample had a corresponding control containing distilled water in place of the carbonyl compound. The time zero sample and control were frozen immediately in liquid nitrogen and stored at −18 °C. A sample and control were removed at each time period and stored until analysis.

During the incubation, many samples formed precipitates or became cloudy. Upon repeated freezing and thawing of the samples, this often became more pronounced. Samples were, therefore, not re-frozen, and analysis was carried out within 1 day.

2.4. Analysis

Wheat protein incubations were analysed by SDS-PAGE as previously described (Gerrard et al., 2001). Lysine analysis required a modification to the OPA method previously used, as described by Bertrand-Harb (Bertrand-Harb, Nicolas, Dalgalavvondo, & Chobert, 1993). OPA was freshly prepared by combining, 25 mL bicine (1.6% w/v), pH 9.4; 40 mg of OPA dissolved in 1 mL methanol, and 100 μL 2-mercaptoethanol. The final volume was adjusted to 50 mL with 1-propanol. Samples were vortexed and heated to 60 °C in a waterbath to aid resolubilisation of the sample, and if necessary, centrifuged at 10,000 *g* for 5 min. Freshly prepared lysine standards, at concentrations between 0.125 and 1 mg/mL, were used to establish calibration curves with each new batch of OPA solution. Albumin and globulin and SDS-insoluble samples were diluted between 1 in 5 and 1 in 10 in order to give results within the linear range of the standard curve. Samples were analysed as described for RNase A, with an OPA solution blank.

Wheat protein concentrations were estimated using a modified version of the Bradford method (Bradford, 1976). Samples were diluted as follows. The albumins and globulins and SDS-insoluble glutenin fractions were diluted 1 in 200, and the gliadin and SDS-soluble fractions to 1 in 100. 300 μL of the diluted sample was then added to a cuvette containing 500 μL of distilled water. Bio-Rad Bradford (200μl) reagent was added and the solution was mixed by covering the cuvette with parafilm and inverting several times. The solution was allowed to incubate at room temperature for 6 min before the absorbance of each sample was measured at

595 nm against a distilled water blank. A standard curve was prepared each day, using bovine serum albumin.

All experiments were repeated in triplicate and each measurement was carried out in triplicate in order to ensure reproducibility and accuracy.

3. Results and discussion

3.1. General

In the previous paper, model studies using ribonuclease as a model protein confirmed the well-established fact that glutaraldehyde crosslinks proteins almost instantaneously. The crosslinking was accompanied by a dramatic loss in the availability of all measurable lysine residues in the protein, confirming that the crosslinking mechanism involves reaction of these amino groups. In contrast, both glyceraldehyde and formaldehyde crosslinked at a lower rate, and removed fewer lysine residues.

3.2. Crosslinking of wheat proteins in vitro—adaptations to the standard methods

Glutaraldehyde, formaldehyde and glyceraldehyde were each incubated with all wheat protein fractions (albumins and globulins, gliadins, SDS-soluble glutenins and SDS-insoluble glutenins) in aqueous solution at 37 °C. Various concentrations of each compound were initially tested. High concentrations of glutaraldehyde were found to crosslink the wheat proteins extremely rapidly. Therefore, in order to obtain information on which protein bands were affected over time, lower doses were employed, with final concentrations of 1 and 10 mM being selected, as they gave the most information on crosslinking. Due to the slower crosslinking action of formaldehyde and glyceraldehyde, a higher dose of 50 mM was used. Freezing and thawing had produced capricious results, due to a detrimental effect on protein solubility; thus each incubation was analysed immediately. The techniques for measurement of protein concentration by the Bradford assay and lysine availability by the OPA method each required modification to accommodate solubility difficulties encountered with the wheat proteins, especially the high molecular weight glutenins; these are detailed in Section 4.

The formation of precipitate occurred to some degree during the incubation of most wheat protein fractions with the carbonyl compounds, indicating that the wheat proteins are not as heat-stable as the model protein, RNase A. Freezing and thawing of the samples exacerbated the solubility problem. To circumvent this, prior to analysis, the samples were briefly heated in a water bath in an attempt to aid the resolubilisation of the precipitate.

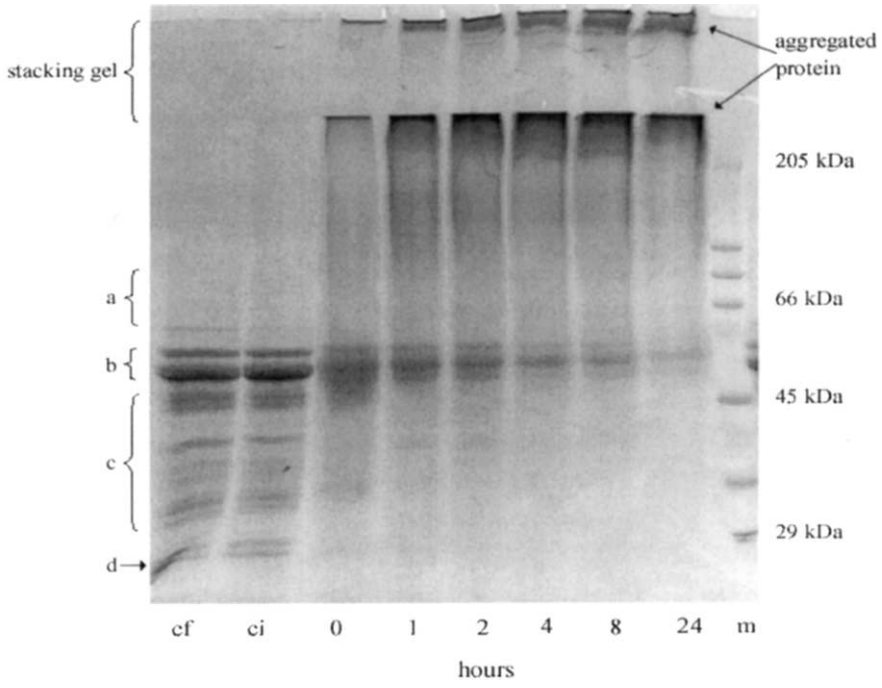

Fig. 1. Typical SDS-PAGE results for the albumin and globulin wheat protein fraction, incubated with 10 mM glutaraldehyde at 37 °C. The frozen control (cf), which was immediately frozen, and the incubated control (ci), which was incubated for 96 h, do not contain glutaraldehyde. The large pore sizes of the stacking gel allow the free migration of all but very large proteins.

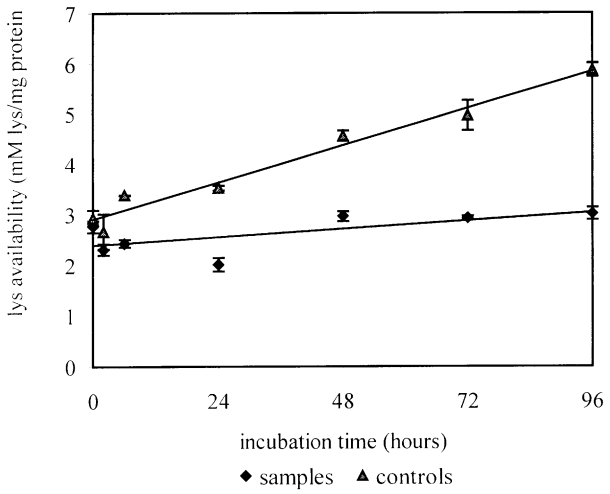

Fig. 2. Typical lysine availability in the albumin and globulin wheat protein fraction when incubated with 1 mM glutaraldehyde at 37 °C. Each point represents the mean of triplicate measurements. Error bars represent the standard error of the mean.

3.3. Crosslinking of wheat proteins by glutaraldehyde in vitro

Upon extraction of the albumin and globulins with dilute sodium chloride, the fraction was observed to form a cloudy solution. Visual inspection of the samples throughout the incubation showed that, after 1 h, the albumin and globulin fraction had started to turn slightly yellow, whilst the controls remained unchanged, indicating that Maillard-type chemistry was occurring. After 10 h, the samples were distinctly yellow, with a white precipitate visible at the bottom of the Eppendorf tube. Precipitation also occurred in the controls, especially after prolonged incubation.

SDS-PAGE results (Fig. 1) show the fading of the protein bands in the regions labelled a–d, with aggregated protein visible, even at time zero. Crosslinks were formed immediately upon addition of glutaraldehyde, before the sample could be frozen in liquid nitrogen. This pattern of crosslinking is entirely consistent with that seen for the ribonuclease model system (preceding paper).

Fig. 2 illustrates a typical lysine count for the albumins and globulins after incubation with 1 mM glutaraldehyde. As expected, the lysine availability of the incubated samples is substantially lower than the controls, consistent with lysine residues having reacted to form protein crosslinks. The overall increasing lysine count was unexpected, but occurred with all the albumin and globulin incubations. This may be explained by the breakdown of some proteins during the incubation. Since OPA reacts with any free amine groups, it also reacts with the N-terminal amine groups revealed by protein breakdown. These N-terminal residues are also available to react with the carbonyl compounds via Maillard chemistry. Thus, two competing reactions may have been occurring, compromising the interpretation of the lysine count data. The presence of proteases in the albumin and globulin fraction could also contribute

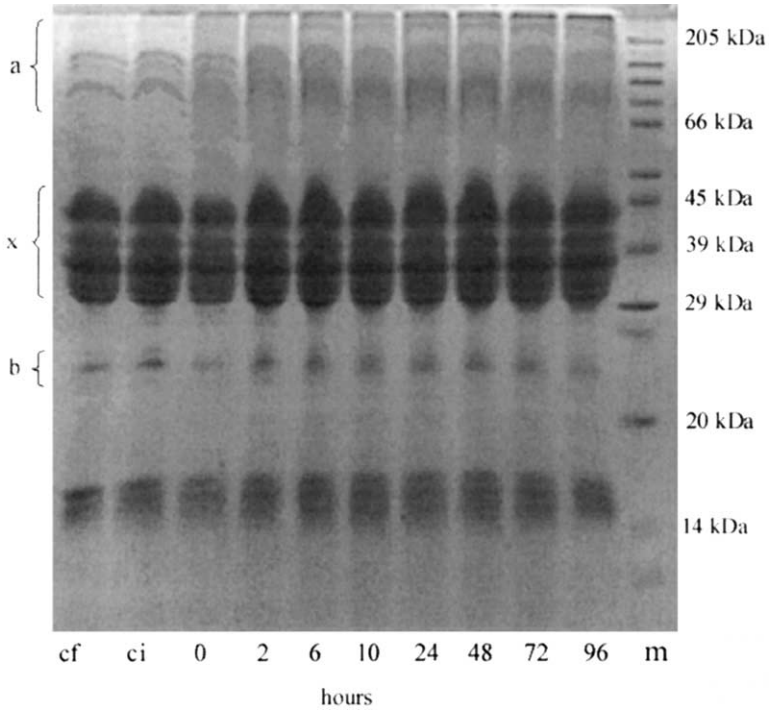

Fig. 3. Typical SDS-PAGE results for the gliadin wheat protein fraction incubated with 1 mM glutaraldehyde at 37 °C. The frozen control (cf), which was immediately frozen, and the incubated control (ci), which was incubated for 96 h, do not contain glutaraldehyde.

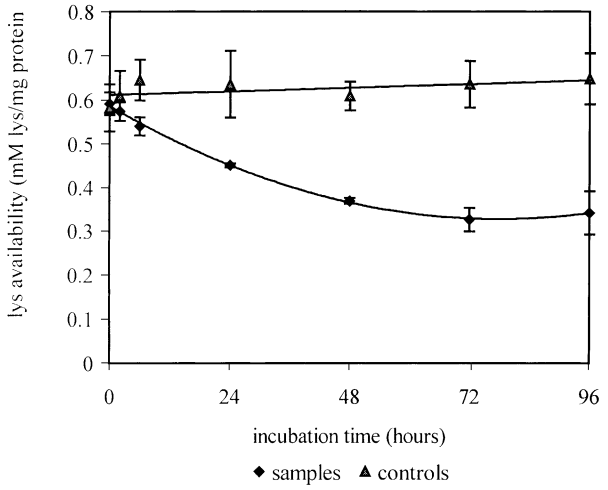

Fig. 4. Typical lysine availability results for the gliadin fraction, incubated with 1 mM glutaraldehyde. Each point represents the mean of triplicate measurements. Error bars represent the standard error of the mean.

to degradation of the protein. These results emphasise the importance of carrying out rigorous control incubations when using 'real food proteins' rather than stable, purified model proteins such as ribonuclease which have been especially selected for their stability.

No colour change was observed during the incubation of glutaraldehyde with the gliadins. The solution remained clear, with a slight precipitate in some samples, upon freezing and thawing, which would disappear upon vortexing. Fig. 3 indicates that some crosslinking had occurred, with a general smearing of protein in region a, and the formation of protein aggregates that are too large to enter the resolving gel. Some fading of the two proteins in region b can also be seen. The bulk of protein in the gliadin fraction labelled x, does not appear to be crosslinked, in contrast to the results seen with both ribonuclease A and the albumin and globulin fraction. Fig. 4 shows a drop in lysine availability, consistent with the occurrence of crosslinking, or reaction of lysine residues that does not result in crosslinking. The gliadin sample appears to be more heat-stable than the albumins and globulin fraction, since the lysine readings for the control samples remain constant.

No colour change was observed to occur when the SDS-soluble fraction was incubated with glutaraldehyde. The freshly extracted SDS-insoluble glutenin fraction contained a white suspension floating on the surface of the buffer, which could not be removed through centrifugation. Lysine analyses of early incubations containing this protein fraction suggested that the protein became more soluble in solution after approximately 12 h. Therefore, in order to optimise incubation conditions, the protein fractions were extracted and stored at room temperature overnight, before the incubations were set up. The SDS-PAGE results, presented in Fig. 5, demonstrate the occurrence of crosslinking, through the fading of protein subunits in the labelled regions, especially in region c, and the aggregation of protein towards the top of the gel, which

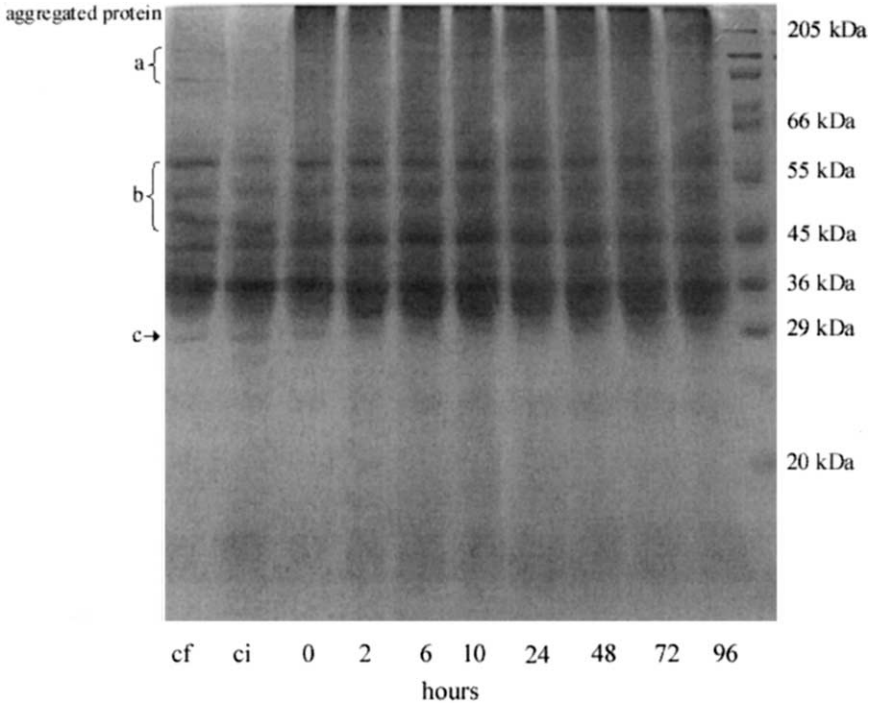

Fig. 5. Typical SDS-PAGE results for the SDS-soluble glutenin fraction, incubated with 1 mM glutaraldehyde at 37 °C. The frozen control (cf), which was immediately frozen, and the incubated control (ci), which was incubated for 96 h, contain no glutaraldehyde.

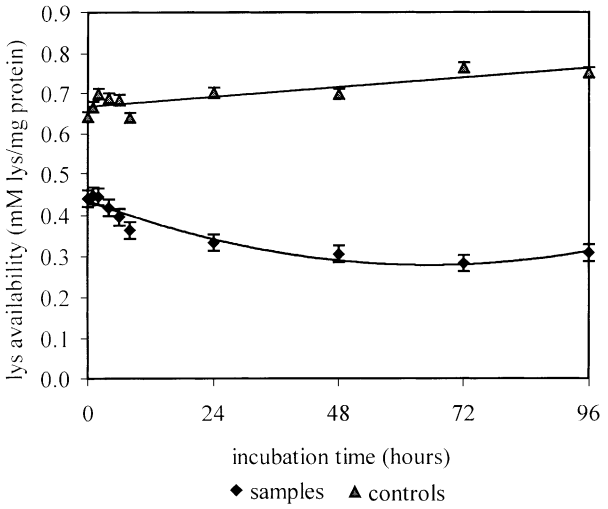

Fig. 6. Typical lysine availability analysis for the SDS-soluble glutenin subunits incubated with 1 mM glutaraldehyde. Each point represents the mean of triplicate measurements. Error bars represent the standard error of the mean..

is not observed in the controls. Lysine determination showed a decrease in lysine availability in the samples incubated with glutaraldehyde, compared to the controls (Fig. 6).

When incubated with glutaraldehyde, the SDS-insoluble glutenins were observed to turn slightly yellow in colour after 1 h; the yellow colour increased in intensity throughout the incubation. The SDS-PAGE results (Fig. 7) clearly show that crosslinking has occurred, with a general fading of bands in the regions labelled a–c, and the immediate appearance of aggregated protein at the top of the gel. The decline in lysine availability (Fig. 8), of the samples compared to the controls, corroborates the electrophoresis results, and affirms that crosslinking began immediately upon addition of the glutaraldehyde.

Thus glutaraldehyde had an immediate action on all four fractions of wheat proteins in vitro, although the reaction was least effective in the gliadin fraction.

3.4. Crosslinking of wheat proteins by glyceraldehyde and formaldehyde in vitro

In contrast to the glutaraldehyde reaction, the glyceraldehyde and formaldehyde crosslinking proceeded at a much slower rate, consistent with our model studies. Glyceraldehyde also caused a yellowing of the wheat proteins, in all but the gliadin incubations. However, with formaldehyde no yellowing of samples was observed, suggesting that formaldehyde may react to form initial products that are not coloured. After 10 h, a white precipitate was observed in the bottom of the tubes. The incubated control, removed at 96 h, was cloudy, whilst the sample remained clear.

The data obtained for glyceraldehyde and formaldehyde were entirely consistent with those of the glutaraldehyde incubation and our model study. SDS-PAGE results obtained for the incubation of formaldehyde with the albumin and globulin fraction are shown in Fig. 9, by way of example. The PAGE gel illustrates immediate crosslinking, with smearing of the proteins and the formation of large aggregated proteins, unable

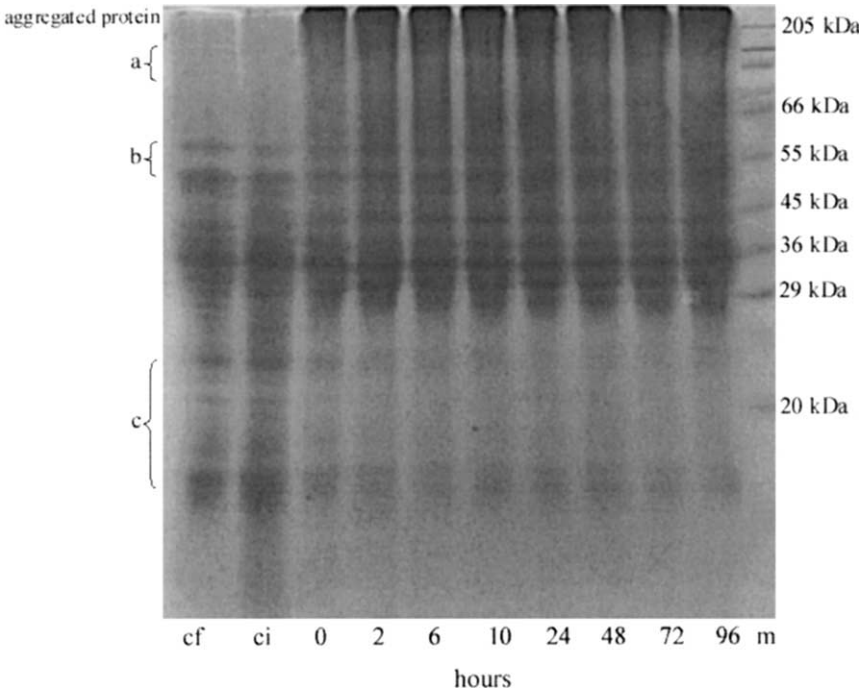

Fig. 7. Typical SDS-PAGE results for the SDS-insoluble glutenins, incubated with 1 mM glutaraldehyde at 37 °C. The frozen control (cf), which was immediately frozen, and the incubated control (ci), which was incubated for 96 h, contain no glutaraldehyde.

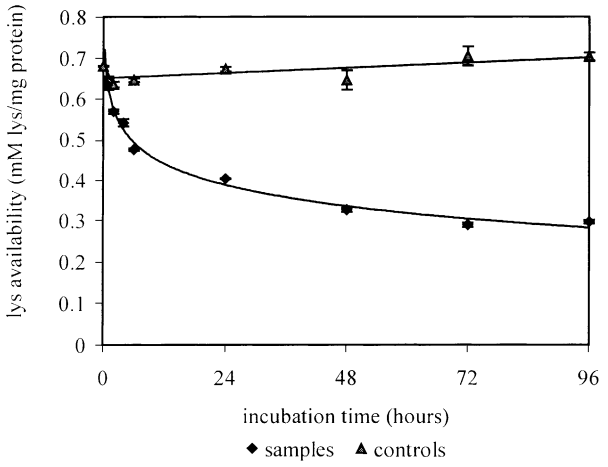

Fig. 8. Typical lysine availability results for the SDS-insoluble glutenins when incubated with 1 mM glutaraldehyde. Each point represents the mean of triplicate measurements. Error bars represent the standard error of the mean.

to enter the stacking and resolving gels. There is general protein loss and blurring of bands in the labelled regions. The proteins running alongside, and slightly ahead of, the 29 kDa marker, that were observed to be involved in crosslinking with glyceraldehyde and glutaraldehyde, appear to remain unchanged when reacted with formaldehyde. This may suggest that the crosslinking reagents have some specificity towards some of the proteins in the albumin and globulin fraction. However, in general, crosslinking appears to occur uniformly, in proportion to the starting concentration of each protein fraction.

3.5. Crosslinking of wheat proteins in situ

To establish whether the in vitro results were relevant to actual food systems, the three crosslinking reagents were each added to bread (and croissant) dough. In order to observe the effect of the crosslinking agents within the dough, doughs containing carbonyl compound at varying concentrations, up to 200 ppm, were made by the standard method. Control doughs, containing no carbonyl compound, were also prepared. The doughs were allowed to rest at 32 °C, with samples being removed and frozen at 30 minute intervals. Protein was extracted from the samples and analysed for protein crosslinking. The effects of the crosslinking reagents on the properties of the dough and on the final baked products are discussed in the following paper.

SDS-PAGE results indicated that, at 100 ppm, none of the carbonyl compounds crosslink wheat proteins within the dough. For doughs treated with 200 ppm glutaraldehyde, crosslinking was evident in the albumin and globulin fraction only, and the reaction was far slowerer than in the in vitro situation (see Fig. 10). Higher doses of crosslinking molecules produced unworkable doughs, as described in the following paper.

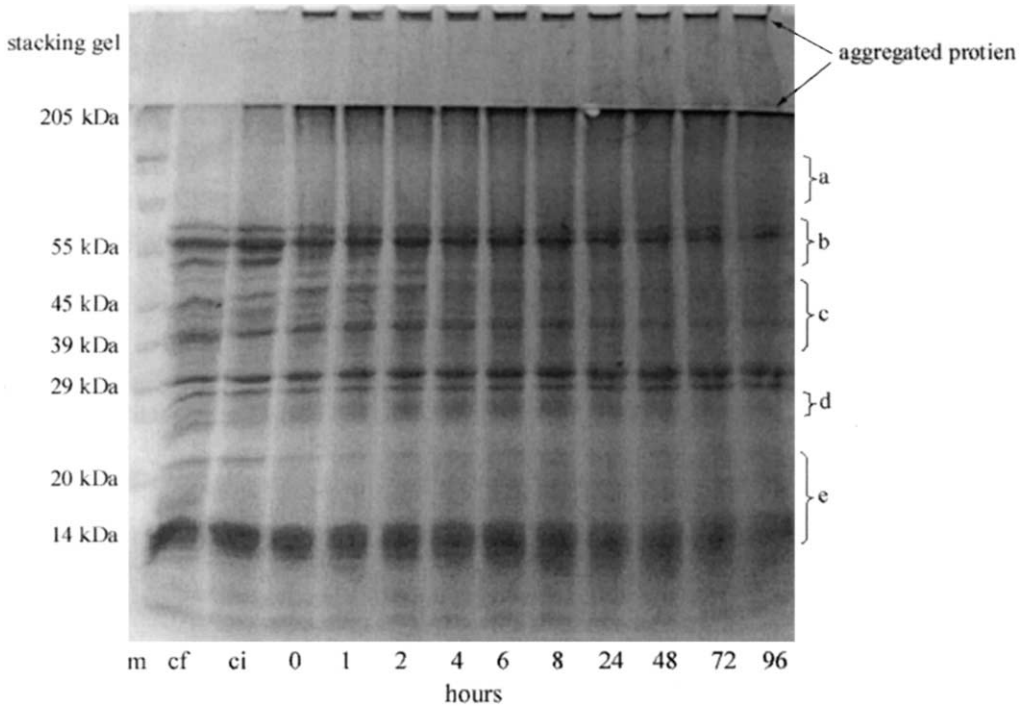

Fig. 9. Typical SDS-PAGE results for the albumin and globulin wheat protein fraction, incubated with 50 mM formaldehyde, at 37 °C. The frozen control (cf), which was immediately frozen, and the incubated control (ci), which was incubated for 96 h, do not contain formaldehyde.

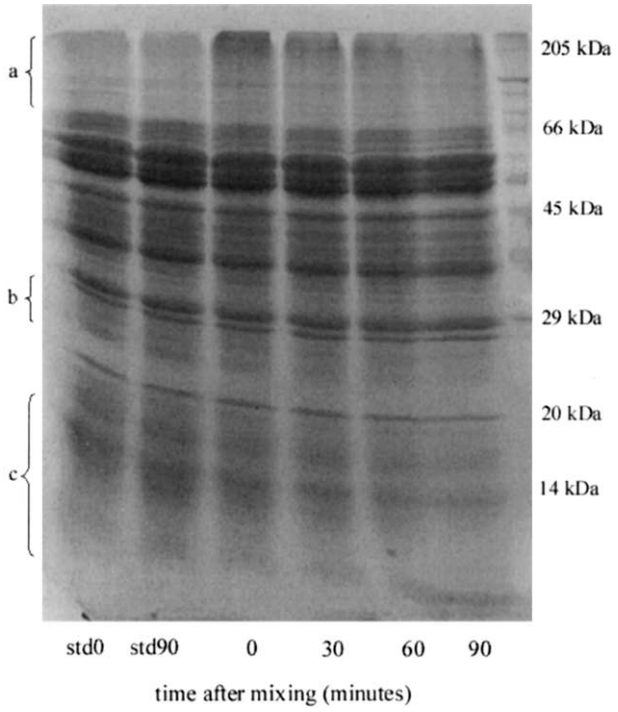

Fig. 10. SDS-PAGE results for the in situ crosslinking of the albumins and globulins mediated by 200 ppm glutaraldehyde.

4. Conclusions

The study of wheat proteins during Maillard crosslinking required some modification of the methods that we had developed to study model proteins. With these methods in hand, four wheat protein fractions (albumins/globulins, gliadins, SDS-soluble glutenins and SDS-insoluble glutenins) were incubated with glutaraldehyde, glyceraldehyde or formaldehyde to assess the ability of these compounds to crosslink wheat proteins via the Maillard reaction. SDS-PAGE analysis clearly showed that each of the three compounds introduced crosslinks into the wheat proteins, although this was not quite so clear when the gliadin fraction was incubated with formaldehyde. In each case, crosslinking was evident through the characteristic fading of protein bands and the corresponding formation of aggregated protein towards the top of the gel. A drop in available lysine was observed in all cases, consistent with crosslinking being through the lysine residues. Interestingly, although the lysine residues of the gliadins appeared to react, very little crosslinking occurs; this suggests some specificity of reaction. Clearly, all three compounds had the potential to crosslink wheat proteins in a dough.

However, when each of the three compounds was added to dough, only high doses of glutaraldehyde were able to crosslink proteins in situ, and only the albumin and globulin fraction was affected. The difference between the reactivity of the proteins in the in vitro and in situ situations emphasises the need to study food proteins, not only in the test-tube, but in the actual food processing situation of interest. Although high doses of the most reactive compound—glutaraldehyde—were needed in order to observe in situ effects, these results are sufficient to demonstrate proof of principle—that Maillard-type crosslinking can occur during food processing. Furthermore, the crosslinking appeared to show some specificity to one group of wheat proteins. The consequences of this specific crosslinking of one fraction of wheat proteins on the properties of bread and croissants are examined in the following paper.

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